Evidence for Two Types of Phosphorylation of Proteins

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The effect of the inhibitors of protein synthesis like ethionine, puromycin and cycloheximide on the incorporation of radioactive amino acids and phosphorus into the proteins of liver, kidney, blood, mammary gland and casein has been studied. The incorporation of the amino acids into the proteins of all these tissues is inhibited in the presence of the inhibitors. The incorporation of the radioactive phosphorus into the proteins of liver and kidney is unaffected; however, that into the proteins of mammary gland is inhibited. No free phosphoserine is detectable in mammary gland. However, further experiments have indicated the presence of phosphoseryl-t-RNA in this tissue. These results indicate the presence of two possible mechanisms of protein phosphorylation, one sensitive to the inhibitors of protein synthesis, occurring in the lactating mammary gland and the other insensitive to these inhibitors and is probably common for other tissues like liver and kidney.

EXTENSIVE work has been carried out in recent years on the synthesis and structure of proteins in general and of conjugated proteins in particular. Among the conjugated proteins, phosphoroteins form a very important group. Considerable investigation in the past about phosphoproteins has indicated their role in various metabolic processes such as enzyme catalysis[1], phosphorylation of membrane bound proteins[2,3], metal ion transfer[2,3], electron transfer[4], energy storage[5] and in cellular regulation[6]. However, the mode of incorporation of inorganic phosphate (Pi) into these proteins is not well understood. Therefore, a study of the phosphorylation of proteins in different tissues was undertaken with special reference to the phosphorylation of proteins in lactating mammary gland as the proteins synthesized in this organ are mainly phosphoproteins. The results presented in this communication reveal that there are two types of phosphorylation of proteins, one sensitive to inhibitors of protein synthesis and occurs in lactating mammary gland and the other insensitive and takes place in liver and kidney.

Experimental Methods

In the *in vivo* experiments, lactating rats at 20 days post-partum were used. The procedure to follow the inhibition of protein synthesis by ethionine was essentially according to the method described by Villa-Travino *et al.*[7]. DL-Ethionine was injected intraperitoneally at a level of 1 mg/g body weight of the rat. Half the dose was administered at zero time. After 2 hr, the second half of the dose was given. Inorganic phosphate-^{32}P ($^{32}P_i$)† or ^{14}C-labelled amino acids were injected intraperitoneally 1 hr after first injection of ethionine. Five hours after the first injection, the animals were sacrificed. Liver, kidney, blood and mammary glands were removed as quickly as possible and chilled in 0·9% saline. From the mammary glands, residual milk was extracted by repeated washing with cold saline[8]. The phosphoproteins from the above organs as well as from milk were prepared by the method described by Freidkin and Lehninger[9]. In one experiment, the casein and phosphoproteins of mammary gland were dissolved in minimum amount of dilute ammonia and dialysed extensively against 0·05*M* phosphate buffer (*p*H 7·0), followed by distilled water and reprecipitated by adjusting the *p*H of the solution to 4·6. No appreciable radioactivity was lost, thereby showing that the phosphate is not loosely bound. Radioactivity of the protein was determined by the use of a Geiger-counter attached to a 'Panex' scaler.

In vitro Experiments using Tissue Slices

Lactating rats at 5 to 10 days post-partum were used. Rats were killed by decapitation. Liver and mammary glands were removed and excised in cold saline. Tissue slices were prepared using 'Stadie Riggs' tissue slicer. Known weight of the slices (about 200 mg) of liver or mammary glands was taken in each of the 50 ml flasks containing 5 ml of the Krebs-Ringer bicarbonate buffer (*p*H 7·5). One set of flasks was used as controls. To another set of flasks were added inhibitors of protein synthesis. All the flasks were saturated with oxygen, closed tightly with rubber stoppers and incubated in a 'Gallenkamp' metabolic shaker at 37°C for 30 min. At the end of incubation, equal volume of 20% trichloroacetic acid (TCA) was added and homogenized. Isolation of phosphoproteins was accomplished by the method described earlier and the radioactivity determined.

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†^{32}P (activity, 10-11 mC/ml) was obtained from Bhabha Atomic Research Centre, Trombay, Bombay. ^{14}C-Amino acids were from Radiochemical Centre, Amersham, UK.

Preparation of ^{32}P-Phosphate Labelled Aminoacyl-t-RNA

Lactating rats at 5 to 10 days post-partum were decapitated. The mammary glands were removed and immediately washed in ice-cold 0·9% saline, minced and were incubated in the presence of medium A (*p*H 7·0) of Keller and Zamecnick[10] containing $^{32}P_i$ for 2 hr at 37°C in an atmosphere of oxygen. After incubation, the reaction mixture was chilled immediately and homogenized using 'Virtis' homogenizer. The isolation and assay of aminoacyl-t-RNA were carried out essentially according to the method described by Moldave[11]. The isolated t-RNA was further purified by dissolving it in cold 0·02*M* acetate buffer (*p*H 5·8) containing 0·1*M* sodium chloride and added dropwise to a column of DEAE cellulose (2×10 cm) previously equilibrated with the same buffer. Elution was performed by a linear gradient of sodium chloride from 0·1*M* to 2*M* in a total volume of 500 ml. Five ml fractions were collected every 15 min. RNA was eluted between the tube numbers 45 to 55. These portions, which were completely devoid of contaminating protein, were pooled and then extracted twice with an equal volume of cold phenol saturated with water and with ether respectively. To the aqueous solution containing RNA, three volumes of 95% ethanol were added and the solution was allowed to stand at −14°C overnight. The precipitate formed was spun down and washed with 80% ethanol dissolved in a few millilitres of cold water, dialysed against cold water overnight and lyophilized.

Results

Ethionine administration appeared to inhibit the incorporation of radioactive phenylalanine and tryptophan into the proteins of liver, kidney, blood, mammary gland and casein (Table 1). However, ethionine did not inhibit the incorporation of $^{32}P_i$ into the proteins of liver, kidney and blood but inhibited $^{32}P_i$ incorporation into casein and the proteins of mammary gland. This experiment was repeated 5 times and similar pattern of results was obtained.

The results presented in Table 2 reveal that 70% of the $^{32}P_i$ from liver and 80 to 100% from mammary gland proteins and casein respectively can be dephosphorylated. This is in accordance with the results reported earlier[8]. The results presented in Table 3 clearly show that on paper electrophorogram the radioactive peak coincided with the protein peak as detected by bromophenol reagent. Free $^{32}P_i$ had a different electrophoretic mobility than that of protein in the electrophorogram.

The results on the effect of ethionine, puromycin and cycloheximide on the incorporation of valine-^{14}C and phosphate-^{32}P are presented in Table 4. It could be seen that all the inhibitors of protein synthesis inhibited the incorporation of valine-^{14}C into liver as well as mammary gland proteins. Ethionine was markedly less effective than puromycin or cycloheximide in inhibiting the *in vitro* incorporation of amino acids. The incorporation of $^{32}P_i$ into liver proteins was not inhibited by any of

TABLE 1 — EFFECT OF INJECTED ETHIONINE ON THE INCORPORATION OF PHENYLALANINE-U-^{14}C, TRYPTOPHANE-7-^{14}C AND PHOSPHATE-^{32}P INTO THE PROTEINS OF VARIOUS ORGANS AND CASEIN OF LACTATING RAT

Source of protein	% decrease in the incorporation of labelled compounds as compared to the control		
	Phenylalanine-U-^{14}C	Tryptophane-7-^{14}C	Phosphate-^{32}P
Liver	33	18	2
Kidney	—	—	1
Blood	52	85	0
Mammary gland	65	30	67
Casein	64	30	74

TABLE 2 — $^{32}P_i$ RELEASE FROM PROTEIN OF LIVER, MAMMARY GLAND AND CASEIN BY BARIUM HYDROXIDE

(Dephosphorylation was carried out at 34°C for 2 hr or 24 hr using 0·05*M* barium hydroxide solution)

	Percentage release of $^{32}P_i$ at	
	2 hr	24 hr
Liver	70	70
Mammary gland	84	84
Casein	85	100

TABLE 3 — ELECTROPHORETIC PATTERN OF CASEIN AND MAMMARY GLAND PHOSPHOPROTEIN

[Electrophoresis was carried out for 3 hr and 30 min using Whatman No. 1 filter paper strips and 0·05*M* veronal buffer (*p*H 8·6) at a voltage gradient of 8·8 V/cm]

	Distance moved by the radioactive peak from the origin (cm)	Distance moved by protein peak from the origin (bromophenol blue test) (cm)
Casein	+2 to 4	+1·5 to 3
Mammary gland phosphoprotein	+3·5 to 5	+2 to 4

TABLE 4 — EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON THE INCORPORATION OF ^{14}C-VALINE AND ^{32}P-PHOSPHATE INTO THE PROTEIN OF LIVER AND MAMMARY GLAND OF THE RAT

(Tissue slices were incubated for 2 hr in experiments with ethionine and 30 min in experiments where puromycin and cycloheximide were used)

Inhibitors of protein synthesis used	Labelled compounds used	% decrease in the incorporation of labelled compounds over the control	
		Liver	Mammary gland
Ethionine (10 mg/ml)	^{14}C-Valine	69·0	29·4
	^{32}P-Phosphate	5·2	10·4
Puromycin (200 μg/ml)	^{14}C-Valine	92·0	88·0
	^{32}P-Phosphate	1·3	19·9
Cycloheximide (200 μg/ml)	^{14}C-Valine	90·0	83·0
	^{32}P-Phosphate	2·8	28·0

the inhibitors of protein synthesis whereas that into mammary gland proteins was inhibited. However, the extent of inhibition of $^{32}P_i$ into mammary gland proteins was almost one-third of inhibition of valine-^{14}C by all these inhibitors of protein synthesis, These results indicated that, at least in mammary gland, there is the possibility of phosphate getting incorporated at a stage earlier to the formation of complete protein, namely (i) phosphorylation of serine, or (ii) phosphorylation of seryl t-RNA. Therefore, experiments were designed to test some of these possibilities.

Presence of Phosphoserine in Rat Mammary Gland

One day old lactating rats were used in these experiments. Mammary glands from normal lactating rats, as well as those given ethionine intraperitoneally as described in ' Methods ', were taken. A 10% homogenate of the washed mammary glands was prepared in cold 10% TCA and centrifuged. TCA-soluble fraction was taken and TCA was removed by ether extraction and the *p*H was adjusted to 7·5-8·0. The sample was loaded on a Dowex-1-C1 (200-400 mesh) column (14×1·5 cm). The column was washed with 200 ml of water and followed by 200 ml of 0·001*N* HCl. Elution was continued with 0·01*N* Hcl, and 5 ml fractions were collected. Eighty fractions were collected and analysed for radioactivity. Of the six radioactive peaks obtained, only two answered ninhydrin test. Tubes 23 to 31 constituted the first peak and those from 32 to 41 formed the second peak. First peak gave intense colour with ninhydrin, whereas the second peak gave faint colour. The two fractions were concentrated and a part of them was chromatographed on Whatman No. 1 filter paper, using 75 parts of ethanol and 50 parts of 0·1*M* acetate buffer (*p*H 4·6) as the solvent system for 24 hr. Ninhydrin colour did not coincide with the radioactivity. The radioactivity was associated with ultraviolet quenching spots. This indicated the absence of free phosphoserine in the mammary gland homogenate either normal or one in which protein synthesis was inhibited by ethionine. This result is in conformation with the earlier observations[12].

Incorporation of Phosphoserine into t-RNA of Rat Mammary Gland

Ten days old lactating rat mammary glands were homogenized in medium A (ref. 6) of *p*H 7·5 containing 0·35*M* sucrose, 0·035*M* $KHCO_3$, 0·025*M* KCl and 0·01*M* $MgCl_2$ and spun at 100000 *g* for 1 hr. The supernatant was adjusted to *p*H 5 with 1*N* HCl. The precipitate formed was centrifuged and, after one wash with 0·15*M* KCl, was dissolved in the above medium. This was used as the enzyme source and contained enough t-RNA to accept amino acids. This enzyme preparation did not release phosphate from either phosphoserine or casein. The incorporation of ^{14}C-serine or ^{32}P-phosphoserine into t-RNA was carried out essentially as described by Moldave[11]. The results presented in Table 5 indicate that both ^{14}C-labelled serine and ^{32}P-labelled phosphoserine can be transferred to t-RNA. The incorporation of amino acid appeared to be very low. This was probably due to the low efficiency (about 4%) of the radioactive counter used.

TABLE 5 — *In vitro* INCORPORATION OF ^{14}C-SERINE AND ^{32}P-PHOSPHOSERINE INTO S-RNA OF LACTATING RAT MAMMARY GLAND

(0·5 μC of serine-3-^{14}C and 0·5 μC of ^{32}P-phosphoserine are added in the incubation mixture as described in results)

		Sp. activity of protein
Serine-^{14}C	Blank	136
	Experimental	256
^{32}P-Phosphoserine	Blank	160
	Experimental	320

Experiments to Demonstrate Phosphoserine in t-RNA Fraction

A 10% homogenate of the lactating mammary glands was prepared in 0·25*M* sucrose. Aminoacyl-t-RNA was prepared from 100000 *g* supernatant by the conventional phenalization method. The preparation was dialysed against water for 18 hr at 5°C. Amino acids were released from aminoacyl-t-RNA by incubating in a medium of *p*H 10·1 for 30 min at 37°C. The contents were dialysed. The dialysate was concentrated and passed through Dowex-1-C1 column. Basic and neutral amino acids were removed from the column by washing with 100 ml of water followed by 100 ml of 0·001*N* HCl. The 0·01*N* eluate was concentrated and analysed by two-dimensional paper chromatography using phenol-water (75: 25, vol./vol.) and butanol-acetic acid-water (4: 1: 1, vol./vol.) as the two solvent systems. Ninhydrin positive spots corresponding to aspartic acid, glutamic acid and phosphoserine could be detected.

This experiment was repeated using ^{32}P-phosphate as tracer. ^{32}P-Phosphate (100 μC/100 g body weight of rat) was injected to rats intraperitoneally. The animals were sacrificed after 2 hr. A 10% homogenate of the washed mammary glands was prepared in 0·25*M* sucrose and phosphoserine was isolated as described above. The spot corresponding to phosphoserine had considerable radioactivity. Further, 0·01*N* HCl eluate from the column which is supposed to contain ^{32}P-phosphoserine was mixed with 10 mg cold phosphoserine and crystallized using ethanol and ether. The crystals were taken in 2 ml of water and chromatographed with ethanol-acetate buffer (*p*H 4·6). The ninhydrin spot contained all the radioactivity.

Although the presence of phosphoserine could be demonstrated in t-RNA fraction, there could be doubt regarding the attachment of phosphoserine to the -CCA terminal of t-RNA molecule specifically as in the case of other amino acids[13]. The following two procedures were used to prove conclusively the presence of phosphoseryl t-RNA in rat mammary gland. The first method was followed essentially according to Preiss *et al.*[14] and the second method according to Soda *et al.*[15].

(i) The aminoacyl-t-RNA isolated from rat mammary gland can be digested with ribonuclease and the aminoacyl adenosine is isolated. The presence of phosphoserine in this fraction will prove the presence of phosphoseryl t-RNA in rat mammary gland. If ^{32}P-phosphate was incorporated into t-RNA, the aminoacyl adenosine would not have nucleotide phosphate. The only major phosphate containing amino acid is phosphoserine under this condition. Thus, the presence of radioactivity in aminoacyl adenosine fraction would prove the presence of phosphoseryl-t-RNA. (ii) The amino acid in an ester bond with t-RNA isolated from rat mammary gland can be reduced using lithium borohydride to amino alcohol. The presence of phosphoserinol in this fraction would prove the presence of phosphoseryl-t-RNA in rat mammary gland.

Mammary gland mince (20 g) was incubated in the presence of phosphate-^{32}P (50 μC) in 40 ml of medium A described under methods. The resulting labelled aminoacyl-t-RNA was isolated, purified and lyophilized according to the procedure described earlier. Lyophilized powder (20 mg) was dissolved in 0·5 ml acetate buffer (*p*H 5·8) and treated with 100 μg of ribonuclease for 15 min at 30°C. An aliquot was placed on Whatman No. 1 filter paper and chromatographed at 4°C with secondary butanol-formic acid-water (70: 10: 20, vol./vol.) as solvent system. The R_f values of serine, phosphoserine and adenosine are represented in Table 6. In comparison with other aminoacyl adenosines, phosphoseryl adenosine was expected to have a R_f value between phosphoserine and adenosine[14]. Following chromatography the strip was cut into 1 cm sections eluted with 0·05*N* NH_4OH and the eluate counted. Almost all the radioactivity was located at the origin and no radioactivity was found at the region where phosphoseryl adenosine was expected. The method followed here has certain limitations in that the R_f value of phosphoseryl adenosine is assumed to be found between phosphoserine and adenosine spots. However, it is possible that phosphoseryl adenosine may not move very far from the origin. Owing to the lack of an authentic sample of phosphoseryl adenosine, it has not been possible to determine the correct R_f value.

TABLE 6 — R_f VALUES OF THE VARIOUS MONONUCLEOTIDES, SERINE, PHOSPHOSERINE, SERINOL, PHOSPHOSERINOL AND ADENOSINE

[Solvent I, *n*-butanol-formic acid-water (70 :10 :20, vol./vol.; solvent II, *tert*-butanol-methyl ethyl ketone-water-concentrated NH_4OH (50 : 50 : 25 : 15, vol./vol.)]

	R_f values	
	Solvent I	Solvent II
Serinol	—	0·87
Phosphoserinol	—	0·32
Serine	0·33	0·32
Phosphoserine	0·12	0·045
Adenosine	0·28	—
AMP, CMP, GMP, UMP	0·04	0·08-0·11

The ^{32}P-labelled aminoacyl-t-RNA was prepared as described above. The lyophilized aminoacyl t-RNA (10 mg) was suspended in 10 ml of 0·15*M* lithium borohydride in tetrahydrofuran (distilled over lithium aluminium hydride) and the mixture was shaken at 26°C for 1 hr. Two ml of 6*N* HCl was added and the solution was heated on a steam-bath until almost all of the tetrahydrofuran had evaporated and the residual solution was neutralized by the addition of 0·1*M* NaOH. The solution was desalted by passing through a column of Dowex-50 (H^+) followed by elution of the radioactive material with 6*N* HCl. The desalted material was concentrated *in vacuo* to dryness over sodium hydroxide until all the HCl was removed. To this residue was added phosphoserinol dissolved in a minimal amount of water and chromatographed on strips of Whatman No. 3 paper using solvent system consisting of *tert*-butanol-methyl ethyl ketone-water-concentrated ammonium hydroxide (50: 50: 25: 15, vol./vol.). After chromatography, the paper strips were cut into 1 cm sections and their radioactivity was determined in a Geiger-Müller counter attached to ' Panex ' scaler. No radioactivity was detected in the region corresponding to phosphoserinol. Other phosphate containing compounds which may be present as contaminants such as nucleotides and phosphoserine had entirely different R_f values as represented in Table 6. This method again has certain limitations, viz. in the isolation of phosphoserinol 6*N* HCl was added before the evaporation of cyclohexanol which may remove considerable phosphate from phosphoserine moiety. Therefore, the results presented in this paper indicate, though not conclusively, the presence of phosphoseryl-t-RNA.

Discussion

Phosphate in casein is present mostly as phosphoserine. Evidences in the literature indicate that phosphate gets into casein at the polypeptide level. This conclusion was based on the following observations: (i) occurrence in many tissues of phosphoprotein kinase which phosphorylates different proteins and not free amino acid, namely serine, and (ii) absence of phosphoserine in mammary gland extracts. However, some of the evidences also suggest that P_i can be incorporated into casein by an entirely different mechanism. For example, (i) phosphoprotein kinases are located in mitochondrial fraction whereas most of the protein synthesis takes place in microsomal fraction, (ii) phosphoprotein kinases phosphorylate among other proteins casein also which is a phosphorylated protein, and (iii) completely dephosphorylated casein could not be phosphorylated by the phosphoprotein kinase. This would mean that phosphoprotein kinase is phosohorylating some other groups on casein molecule, suggesting that *in vivo* phosphorylation is different from that of *in vitro* phosphorylation. Further, phosphoserine can be formed not necessarily from phosphorylation of serine but also from phosphoenol pyruvic acid. It is also possible that phosphorylation of serine can take place at the t-RNA level. From these observations it is conceivable that phosphate gets incorporated into

casein before the peptide bond is formed either at the amino acid level or at the t-RNA stage. Carlson *et al.*[16] reported the occurrence of phosphoseryl-t-RNA in hen's oviduct. Further data presented in this communication indicate that ethionine, an inhibitor of *in vivo* protein synthesis, inhibits *in vivo* incorporation of both the amino acid and phosphate into casein to the same extent (Table 1), indicating thereby that P_i gets incorporated into casein before the peptide bond is formed. However, in the case of liver ethionine did not inhibit the incorporation of $^{32}P_i$ into proteins under conditions when it inhibited the amino acid incorporation to a significant extent, suggesting that in liver phosphoproteins P_i may get into the preformed proteins. It can also be argued that decreased $^{32}P_i$ incorporation into mammary gland proteins by ethionine may be due to lack of ATP, because it has been shown that ethionine administration decreases hepatic ATP[17]. Ethionine administration along with adenine had no effect on the hepatic ATP levels. However, in a couple of experiments, ethionine, when administered along with adenine, had no effect on the decreased incorporation of ^{32}P-phosphate into mammary gland proteins. Further, the results presented in Table 1 indicate that ethionine has no effect on the incorporation of radioactive phosphorus into the proteins of liver, though the ATP levels of this tissue are known to be low under these conditions[17].

Puromycin, cycloheximide and ethionine, when tested under *in vitro* conditions using tissue slices, inhibited the incorporation of amino acid and not of ^{32}P-phosphate into liver proteins. However, in mammary gland slices, they inhibited both amino acid and $^{32}P_i$ incorporations into proteins. Unlike the *in vivo* results, the incorporation of $^{32}P_i$ into proteins is not inhibited to the same extent as that of amino acid incorporation. This lack of maximal inhibition may be ascribed to the phosphorylation of groups other than that to be phosphorylated under *in vivo* conditions which step may not be sensitive to these inhibitors.

When the investigation was in progress, Turkington and Topper[18], using mouse mammary explants, showed that phosphorylation was relatively insensitive to the presence of puromycin during the first 2 hr of incubation and as the incubation proceeded further the rate of incorporation of ^{32}P in the presence of puromycin was the same as that of the ^{14}C-amino acid incorporation. Further, singh *et al.*[19] showed that in rat mammary gland slices the inhibition of ^{32}P-phosphate incorporation by puromycin was only at later time intervals. However, in our experiments, using puromycin and cycloheximide, we obtained consistently significant inhibition in 30 min. Although we did not prove whether phosphorylation was taking place before the complete protein was formed, i.e. at the amino acid or t-RNA stage, but revealed the existence of two types of phosphorylation, one sensitive to the inhibitors of protein synthesis and occurring in mammary gland and the other insensitive to these inhibitors of protein synthesis and occurring in liver. The later phenomenon has also been shown in thymus nuclei by Kleinsmith *et al.*[6].

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